Reference: *Biol. Bull.* **183:** 78–83. (August, 1992)

Localization of Laminin in the Subepidermal Basal Lamina of the Planarian *Dugesia japonica*

ISAO HORI

Department of Biology, Kanazawa Medical University, Uchinada, Ishikawa 920-02, Japan

Abstract. The planarian subepidermal basal lamina consists of three structural elements: namely, an electronlucent zone, a limiting layer, and a microfibrillar layer. Ultrastructural observations following ruthenium red staining were in agreement with previously published reports. The staining clearly revealed positive material on the limiting layer and the individual microfibrils. The limiting layer was continuous and separated the electronlucent zone from the microfibrillar layer. The distribution of laminin, a noncollagenous basal lamina glycoprotein, was determined for the planarian basal lamina using various methods of immunocytochemistry. The reactive substance appeared only on the limiting layer and in the electronlucent zone along the limiting layer. The reactive produets on the limiting layer appeared discontinuous. They were also present in the regenerated basal lamina, though the reactivity was a little weaker than in that of intaet animals. Localization of laminin in the planarian subepidermal basal lamina is discussed in comparison with that of the basal lamina of vertebrates.

Introduction

The basal lamina or basement membrane is responsible for the maintenance of epithelial integrity, and its condition determines cell behavior during wound repair (Schittny *et al.*, 1988). Consequently, a variety of studies have been carried out with regard to each component of the basal lamina. In view of current knowledge, its molecular components are type IV collagen, sulfated proteoglycans, fibronectin, and laminin, and their distribution can be associated with the function of the epithelial cells (Farquhar, 1981; Laurie *et al.*, 1982; Newgreen, 1984; Simo *et al.*, 1991).

Received 21 October 1991; accepted 18 May 1992.

The subepidermal basal lamina of planarians is structurally quite different from that of higher animals. The planarian basal lamina is characterized by an electronlucent zone of irregular shape and a microfibrillar layer (Pedersen, 1966; Bedini and Papi, 1974; Rieger, 1981; Tyler, 1984). Extracellular matrix components are concentrated mainly in the subepidermal basal lamina. Our previous studies using electron microscopy and autoradiography have shown that the basal lamina is regenerated by interaction between the wound epidermis and differentiating myoblasts (Hori, 1979, 1980). Although the morphologieal ehanges and behavior of regenerative eells have been studied well in planarians, cytochemical information about its molecular components is not readily available. This study examines by immunocytochemistry the localization of laminin, a kind of extracellular glycoprotein, in the planarian basal lamina and compares it with the results of ruthenium red staining.

Materials and Methods

Animals

The freshwater planarian *Dugesia japonica* was used in this study. Healthy worms (10–15 mm in length) were selected and maintained without food for a week in our laboratory. Intact tissues from the region between the auricles and pharynx were dissected from six worms. Regenerates of six other worms were obtained by decapitation. Specimens were allowed to regenerate for 4 or 6 days in tap water at 18°C.

Electron microscopy for ruthenium red staining

For the detection of extracellular proteoglycans, tissues were processed for ruthenium red (RR) staining according to Luft (1971). They were fixed for 60 min in 1.2% glutaraldehyde at 4°C, and postfixed for 3 h in 1% osmium

tetroxide at room temperature. Both fixatives were buffered with 0.1 *M* sodium cacodylate (pH 7.4) containing 0.1% ruthenium red (TAAB). Fixation was carried out in a dark room. Specimens were dehydrated with a graded ethanol series and embedded in Epon 812. Thin sections were counterstained with uranyl acetate and lead citrate, and then photographed with a Hitachi H-500 electron microscope.

Immunohistochemistry

Tissues were fixed in Zamboni fixative (Zamboni and DeMartino, 1967) for 4 h at 4°C. After being rinsed for 30 min with cold TBS (pH 7.5), they were dehydrated with graded ethanols and embedded in paraffin. The immunoreaction was carried out according to the AB Complex method (Hsu *et al.*, 1981). Deparaffinized sections (3–5 μm thick) were incubated for 5 min with 3% hydrogen peroxide and incubated for 20 min with bovine serum (normal) diluted 1:5 in TBS. The sections were then incubated overnight at 4°C with rabbit anti-laminin antibody (Sanbio) diluted 1:50 in TBS. For controls, sections were incubated with TBS. All the sections were then incubated for 30 min with biotinylated swine anti-rabbit immunoglobulins (DAKO) diluted 1:300 in TBS, and then again for 30 min with avidin and biotinylated horseradish peroxidase reagents (AB Complex/HRP, DAKO). After being rinsed with TBS they were incubated for 5 min with 3,3 diaminobenzidine tetra-hydrochloride (DAB).

Tissue processing for immunoelectron microscopy

Tissues were fixed for 2 h in 1% glutaraldehyde and 2% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.4) at 0°C. Tissue pieces were rinsed for 30 min in the buffer, dehydrated in graded ethanols at progressively lowered temperature (down to −25°C), and embedded in Lowicryl HM20 according to Carlemalm *et al.* (1982). The samples were transferred to pure resin at −35°C and maintained overnight. Capsules filled with fresh precooled resin were polymerized for at least 24 h under UV light at −35°C. They were then further hardened at room temperature for 2 days. Thin sections were mounted on collodion-supported nickel grids and immunostained by the following two methods.

(1) ABC method. The sections were preincubated for 30 min with TBS containing 1% bovine serum albumin (BSA). Then they were incubated overnight with polyclonal rabbit anti-laminin antibody (Sanbio) diluted 1:50–1:200 in TBS at 4°C. For controls, sections were incubated with TBS. After being rinsed with TBS, they were incubated for 30 min with affinity-isolated, biotinylated swine anti-rabbit immunoglobulins (DAKO) diluted 1:300 in TBS, followed by incubation with AB Complex/HRP for 2 h. Then they were treated for one minute with DAB. After being rinsed with TBS and distilled water, they were observed without counterstaining.

(2) PAG method. Another group of sections were immunostained by Protein A-gold (PAG) reagents according to Roth *et al.* (1978). Thin sections were rinsed for 5 min with PBS and preincubated for 20 min with PBS BSA (1%). They were then incubated overnight with polyclonal rabbit anti-laminin antibody (Sanbio) diluted 1:50–1:500 in PBS BSA at 4°C. After being rinsed with PBS, they were incubated for 45 min with Protein A-gold colloids (Funakoshi) diluted 1:10 in PBS. They were rinsed with PBS and distilled water and stained with uranyl acetate and lead citrate.

Results

Morphological aspects of the basal lamina

The general architecture of the subepidermal basal lamina stained with ruthenium red is shown in Figure 1. The basal lamina separates the single-layered epidermis from underlying muscle fibers. It is divided into three structural elements: namely, an electronlucent zone surrounded by the basal cytoplasmic processes of epidermal cells, a microfibrillar layer including a number of microfibrils, and a limiting layer separating these elements.

The electronlucent zone changes its shape according to basal alterations of the epidermal cells. In most areas, specific filaments are seen producing a meshwork within this zone. In the cross-sectioned basal lamina, these filaments often run parallel to the limiting layer (Fig. 1). The limiting layer is linear and uniform, identical to the lamina densa of the vertebrate basal lamina. The limiting layer had a strong affinity for RR (Fig. 1), and this dye revealed the continuous nature of the layer. Each basal process of an epidermal cell characteristically develops a hemidesmosome at its tip, and the epidermal cells come in contact with the limiting layer through such hemidesmosomes. When RR-treated specimens were viewed at a higher magnification, the RR-positive material was particularly evident at the hemidesmosomal regions (Fig. 1, inset). The microfibrillar layer constitutes most of the basal lamina. It is underlain with plasma membranes of muscle cells. Its thickness varies from 1 to 4 μm. Viewed in cross section, most of microfibrils were coated with RR-positive material (Fig. 1, inset).

The cytoplasmic portions of some kind of parenchymal gland cells are often seen intruding into the epidermal layer. Therefore their cytoplasmic portions, including secretory granules, can also be seen within the microfibrillar layer (Fig. 1).

Immunohistochemical observations

The distribution of laminin was first investigated at the light microscopic level by indirect immunohistochem-

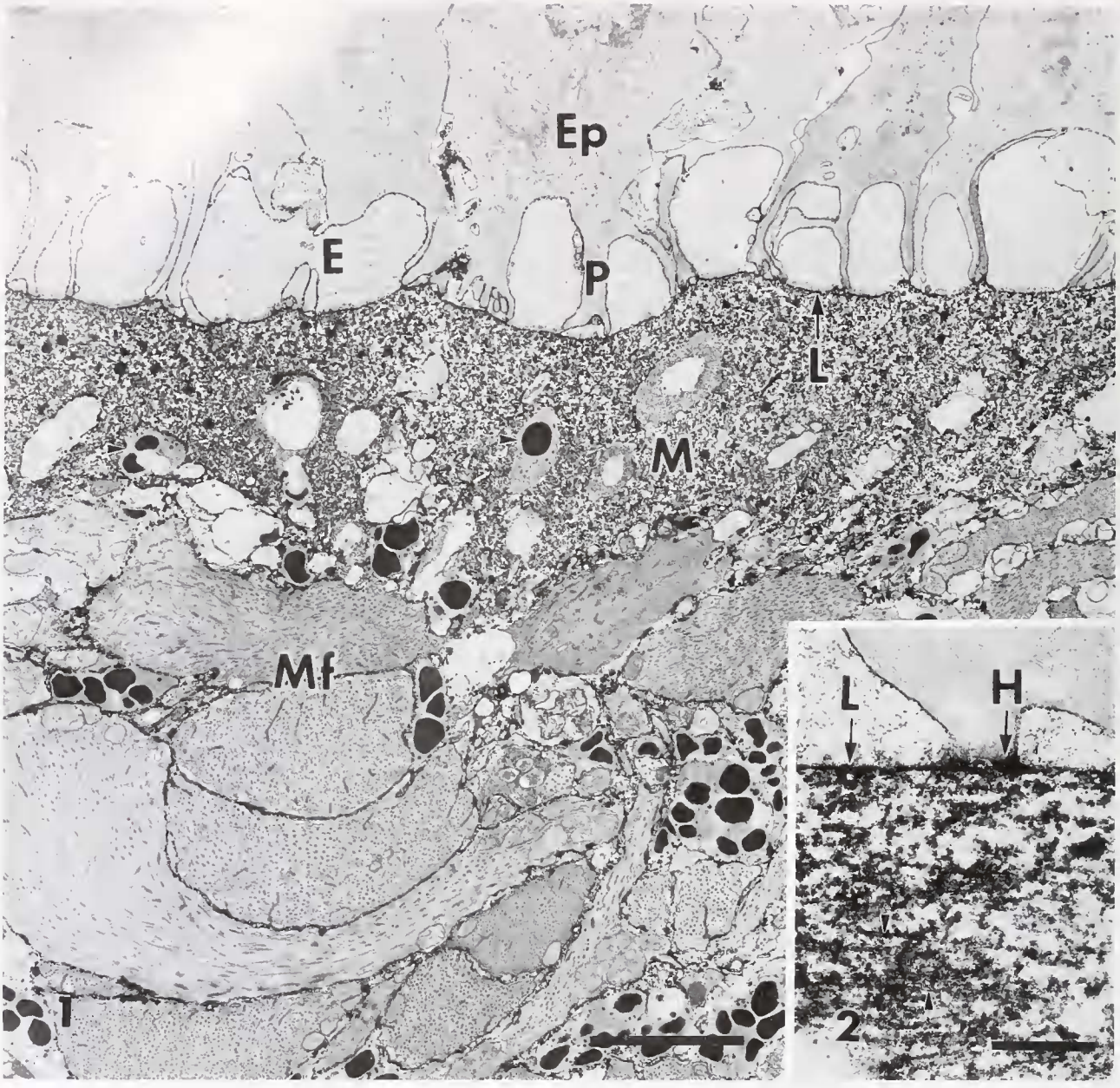
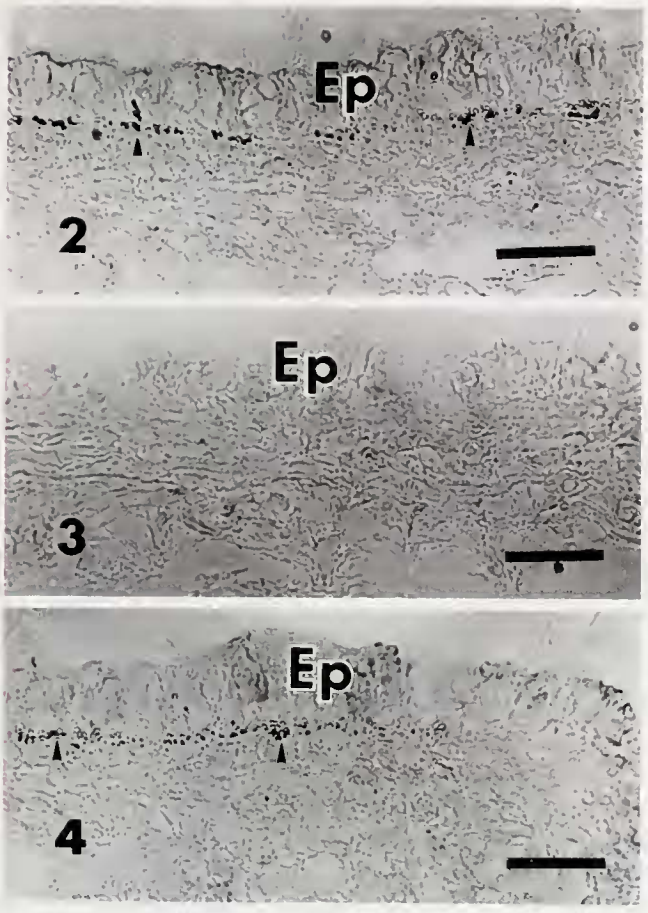


Figure 1. Low magnification view to demonstrate ruthenium red-positive areas in the subepidermal basal lamina. Three structural elements of the basal lamina are evident. Arrowheads indicate the cytoplasmic portion of a gland cell. Scale bar = 2 μm. Inset: High magnification view of the hemidesmosomal region. Ruthenium red staining. The limiting layer is stained clearly. Arrowheads indicate positive material surrounding microfibrils. Scale bar = 0.5 μm. Abbreviations: Ep, epidermal cell; P, cytoplasmic process; E, electronlucent zone; L, limiting layer; M, microfibrillar layer; Mf, muscle fiber; H, hemidesmosome.

istry. Dense deposits, indicating binding of the antibody to laminin, were observed on the basal lamina. The deposits were especially prominent on its epidermal side (Fig. 2). Similar deposits were also seen in the same region of the 6-day regenerate (Fig. 4). The control sections incubated with TBS showed no reactive deposits in the basal lamina (Fig. 3).

Immunoelectron microscopic observations

To resolve the localization of laminin in the basal lamina, immunoelectron microscopy was applied to Lowicryl-embedded thin sections. The ABC method showed dense immunoreactivity on the limiting layer and on a part of the electronlucent zone along the limiting layer (Fig. 5).



Figures 2–4. Immunohistochemical localization of laminin in the basal lamina. Reactive products are distributed along the basal lamina (arrowheads). Scale bar = 20 μm. **Figure 2.** Intact. **Figure 3.** Intact; control. **Figure 4.** Six-day regenerate. Ep, epidermal cell.

In control sections, no reactive substances were seen in the basal lamina (Fig. 6). The stained material on the limiting layer appeared discontinuous. In the 6-day regenerate, when newly formed basal lamina appeared, the reactivity of the limiting layer was also evident, though the staining was slightly weak (data not shown). In both cases, there were no reactive products in the microfibrillar layer.

The localization of laminin in the basal lamina of intact and regenerating worms was compared by the PAG method. The labeling of laminin resulted in significant deposits in the limiting layers of both intact and regenerated tissues (Figs. 7, 8). Fewer gold particles were in the microfibrillar layer than in the limiting layer.

Discussion

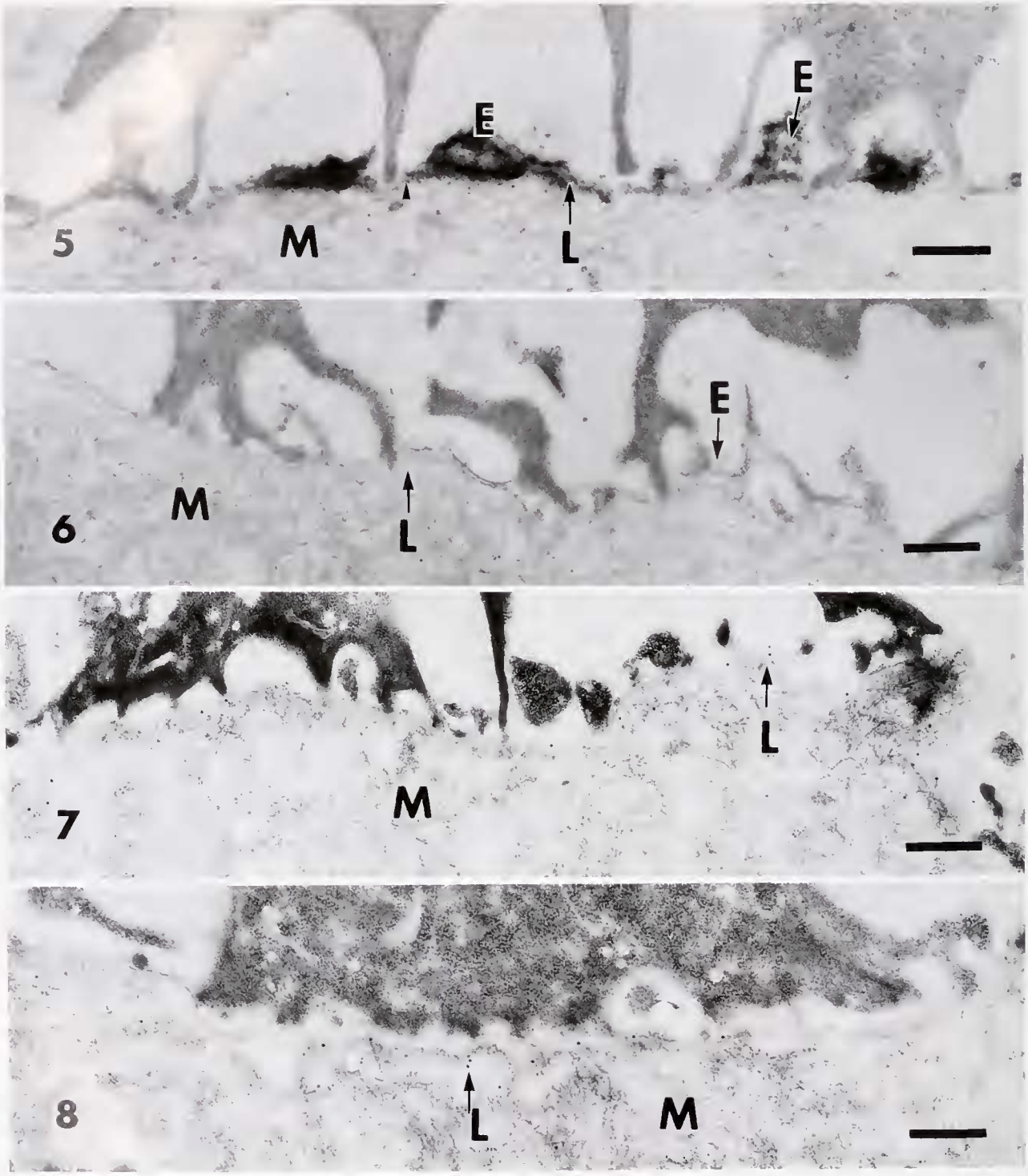
The planarian subepidermal basal lamina has varying degrees of development in different species (Bedini and Papi, 1974). The occurrence and constitution of the planarian basal lamina may not reflect the phylogenetic position of animals, but rather unique functional significance of the basal lamina (Lindroos, 1991). Variations in thickness mainly relate to the extent to which the microfibrillar layer is developed (Sluys, 1989). For example, Rhabdocoela have no microfibrils (Holt and Metrick, 1975), whereas some species of marine triclad have well-organized microfibrillar layer (MacRae, 1965). The basal lamina of the former is similar to that of mammals, and the basal lamina of the latter is similar to that of amphibian larvae (Hay and Revel, 1963). In spite of such variations, the limiting layer can be seen in the basal laminae of most turbellarians.

The results of RR staining provide additional information about the structure and chemical nature of the basal lamina. Because we could demonstrate the RR-positive material in the planarian basal lamina, the limiting layer probably includes sulfated proteoglycans. This is in agreement with the results of experiments on human basement membranes (Horiguchi *et al.*, 1989). The electronlucent zone is occupied by reticular filaments. In our samples, these filaments appear as a meshwork running parallel to the limiting layer. Such a meshwork is, however, not always seen in the planarian basal lamina (Skaer, 1961; Rieger, 1981). The variation in the structure of reticular filaments may depend, not only on their chemical properties, but also on methods of tissue preparation.

Laminin is one of the main components of basal laminae. Its distribution has been examined in many vertebrates (Jacob *et al.*, 1991). Because the antigenic determinants of laminin are not species-specific (Foidart *et al.*, 1980), we have used rabbit anti-laminin antibodies to detect the localization of laminin in the planarian basal lamina. Immunohistochemical staining demonstrated that the distribution of laminin is restricted to the limiting layer and a part of the electronlucent zone. In contrast to the RR-positive proteoglycans, laminin is distributed on the limiting layer discontinuously. This finding is in accord with the observations of Lindroos and Still (1988), who noted irregular deposits of laminin beneath the epithelium of *Polycelis nigra*.

The localization of laminin on vertebrate basement membranes differs from one report to the next: *i.e.*, it is localized in the entire region of the basal lamina (Inoué, 1989); in the lamina densa (Laurie *et al.*, 1982); in the lamina lucida (Foidart *et al.*, 1980; Madri *et al.*, 1980); and at the junction between the lamina densa and lamina lucida (Schittny *et al.*, 1988). Laminin is known to play many important roles in various phenomena, but nothing has been reported about the significance of such varied distributions.

Planarian epidermal cells have no ability to proliferate mitotically so that certain parenchymal cells migrate into the epidermis to produce its cellular succession. In my



Figures 5–6. Localization of laminin by ABC method of Lowicryl-embedded sections. The limiting layer and a part of the electronlucent zone are stained. Arrowhead indicates a discontinuous portion of reactive products. **Figure 5.** Intact. Scale bar = 0.5 μm. **Figure 6.** Control. Scale bar = 0.5 μm.

Figures 7–8. Localization of laminin by immunogold labeling of Lowicryl-embedded sections. Gold particles are seen associated with the limiting layer. **Figure 7.** Intact. Scale bar = 1 μm. **Figure 8.** Four-day regenerate. Scale bar = 0.5 μm. E, electronlucent zone; L, limiting layer; M, microfibrillar layer.

previous study, I ascertained that rhabdite-forming cells are differentiated from regenerative cells and contribute to the cellular succession, both in intact and regenerating planarians (Hori, 1978). Moreover, other kinds of parenchymal gland cells usually extend their cytoplasmic processes, which contain secretory granules, into the epidermal layer (Tyler, 1984). Thus one can assume that the planarian basal lamina probably provides a microenvironment that guides such cell movement from the parenchyma to the epidermis. Recent studies suggest that changes of laminin accumulation affect various cell behaviors, such as cell movement (Simo *et al.*, 1991), cell differentiation (Kubota *et al.*, 1988), interaction between epithelial and underlying cells (Richoux *et al.*, 1989), and cell proliferation (Hogan, 1981). Planarian regeneration is also a complex process including extracellular matrix components. In an earlier study, I reported that fibronectin, another extracellular glycoprotein, is detected around migrating cells within the planarian blastema (Hori, 1991), but the data were based only on the immunocytochemistry of fixed and resin-embedded tissues. Further analysis will require that experiments be carried out *in vitro* to examine the roles of such glycoproteins in cell behavior during planarian regeneration.

Literature Cited

Bedini, C., and F. Papi. 1974. Fine structure of the turbellarian epidermis. Pp. 108–147 in *Biology of Turbellaria*, N. W. Riser, and M. P. Morse, eds. McGraw-Hill Book Co., New York.

Carlemalm, E., R. M. Garavito, and W. Villinger. 1982. Resin development for electron microscopy and an analysis of embedding at low temperature. *J. Microsc.* **126:** 123–143.

Farquhar, M. G. 1981. The glomerular basement membrane: a selective macromolecular filter. Pp. 335–378 in *Cell Biology of Extracellular Matrix*, E. D. Hay, ed. Plenum Press, New York.

Foidart, J. M., E. W. Bere, M. Yaar, S. I. Rennard, M. Gullino, G. R. Martin, and S. I. Katz. 1980. Distribution and immunoelectron microscopic localization of laminin, a noncollagenous basement membrane glycoprotein. *Lab. Invest.* **42:** 336–342.

Hay, E. D., and J. P. Revel. 1963. Autoradiographic studies of the origin of the basement lamella in *Ambrystoma. Dev. Biol.* 7: 152–168.

Hogan, B. 1981. Laminin and epithelial cell attachment. *Nature* **290:** 737–738.

Holt, P. A., and D. F. Mettrick. 1975. Ultrastructural studies of the epidermis and gastrodermis of *Syndesmis franciscana* (Turbellaria; Rhabdocoela). *Can. J. Zool.* **53:** 536–549.

Hori, I. 1978. Possible role of rhabdite-forming cells in cellular succession of the planarian epidermis. *J. Electron Microsc.* **27:** 89–102.

Hori, I. 1979. Structure and regeneration of the planarian basal lamina: an ultrastructural study. *Tissue Cell* **11:** 611–621.

Hori, I. 1980. Localization of newly synthesized precursors of basal lamina in the regenerating planarian as revealed by autoradiography. *Tissue Cell* **12:** 513–521.

Hori, I. 1991. Role of fixed parenchyma cells in blastema formation of the planarian *Dugesia japonica*. *Int. J. Dev. Biol.* **35:** 101–108.

Horiguchi, V., J. R. Couchman, A. V. Ljubimov, H. Yamasaki, and J-D. Fine. 1989. Distribution, ultrastructural localization, and ontogeny of the core protein of a human sulfate proteoglycan in human skin and other basement membranes. *J. Histochem. Cytochem.* **37:** 961–970.

Hsu, S-M., L. Raine, and H. Fanger. 1981. A comparative study of the peroxidase-antiperoxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. *Am. J. Clin. Pathol.* **75:** 734–738.

Inoué, S. 1989. Ultrastructure of basement membranes. *Int. Rev. Cytol.* **117:** 57–98.

Jacob, M., B. Christ, H. J. Jacob, and R. E. Poelmann. 1991. The role of fibronectin and laminin in development and migration of the avian Wolffian duct with reference to somitogenesis. *Anat. Embryol.* **183:** 385–395.

Kubota, Y., H. K. Kleinman, G. R. Martin, and T. J. Lawley. 1988. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J. Cell Biol.* **107:** 1589–1598.

Laurie, G. W., C. P. Leblond, and G. R. Martin. 1982. Localization of type IV collagen, laminin, heparan sulfate proteoglycan, and fibronectin to the basal lamina of basement membranes. *J. Cell Biol.* **95:** 340–344.

Lindroos, P. 1991. Aspects on the extracellular matrix and protonephridia in flatworms, with special reference to the tapeworm *Diphyllobothrium dendriticum*. Pp. 6–53 in Thesis. Åbo Akademis Kopieringscentral, Åbo.

Lindroos, P., and M. J. Still. 1988. Extracellular matrix components in *Polycelis nigra* (Turbellaria, Tricladida). *Fortsch. Zool.* **36:** 157–162.

Luft, J. H. 1971. Ruthenium red and violet II. Fine structural localization in animal tissues. *Anat. Rec.* **171:** 369–416.

MacRae, E. K. 1965. Fine structure of the basement lamella in a marine turbellarian. *Am. Zool.* **5:** 247.

Madri, J. A., F. J. Roll, H. Furthmayr, and J. M. Foidart. 1980. Ultrastructural localization of fibronectin and laminin in the basement membranes of the murine kidney. *J. Cell Biol* **86:** 682–687.

Newgreen, D. 1984. Spreading of explants of embryonic chick mesenchymes and epithelia on fibronectin and laminin. *Cell Tissue Res.* **236:** 265–277.

Pedersen, K. J. 1966. The organization of the connective tissue of *Discocelides langi* (Turbellaria, Polycladida). *Z. Zellforsch.* **71:** 94–117.

Richoux, V., T. Darribère, J-C. Boucaut, J. E. Elèchon, and J-P. Thiery. 1989. Distribution of fibronectins and laminin in the early pig embryo. *Anat. Rec.* **223:** 72–81.

Rieger, R. M. 1981. Morphology of the turbellaria at the ultrastructural level. *Hydrobiologia* **84:** 213–229.

Roth, J., M. Bendayan, and L. Orci. 1978. Ultrastructural localization of intracellular antigens by the use of protein A-gold complex. *J. Histochem. Cytochem.* **26:** 1074–1081.

Schittny, J. C., R. Timpl, and J. Elgel. 1988. High resolution immunoelectron microscopic localization of functional domains of laminin, nidogen, and heparan sulfate proteoglycan in epithelial basement membrane of mouse cornea reveals different topological orientations. *J. Cell Biol.* **107:** 1599–1610.

Simo, P., P. Simon-Assmann, F. Bouziges, C. Leberquier, M. Kedinger, P. Ekblom, and L. Sorokin. 1991. Changes in the expression of laminin during intestinal development. *Development* **112:** 477–487.

Skaer, R. J. 1961. Some aspects of the cytology of *Polycelis nigra*. *Q. J. Microsc. Sci* **102:** 295–317.

Sluys, R. 1989. *A Monograph of the Marine Triclads*. Pp. 1–5. A. A. Belkema, Rotterdam.

Tyler, S. 1984. Turbellarian platyhelminths. Pp. 112–132 in *Biology of the Integument Vol. 1 Invertebrates*, J. Bereiter-Hahn, ed. Springer-Verlag, Berlin.

Zamboni, L., and C. DeMartino. 1967. Buffered picric acid-formaldehyde: a new, rapid fixative for electron microscopy. *J. Cell Biol.* **35:** 148A.